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Changes in Gene Expression within the Extended Amygdala following Binge-Like Alcohol Drinking by Adolescent Alcohol-Preferring (P) Rats

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Abstract

The objective of this study was to determine changes in gene expression within the extended amygdala following binge-like alcohol drinking by male adolescent alcohol-preferring (P) rats. Starting at 28 days of age, P rats were given concurrent access to 15 and 30 % ethanol for 3 one-h sessions/day for 5 consecutive days/week for 3 weeks. Rats were killed by decapitation 3 h after the first ethanol access session on the 15th day of drinking. RNA was prepared from micropunch samples of the nucleus accumbens shell (Acb-sh) and central nucleus of the amygdala (CeA). Ethanol intakes were 2.5 – 3.0 g/kg/session. There were 154 and 182 unique named genes that significantly differed (FDR = 0.2) between the water and ethanol group in the Acb-sh and CeA, respectively. Gene Ontology (GO) analyses indicated that adolescent binge drinking produced changes in biological processes involved with cell proliferation and regulation of cellular structure in the Acb-sh, and in neuron projection and positive regulation of cellular organization in the CeA. Ingenuity Pathway Analysis indicated that, in the Acb-sh, there were several major intracellular signaling pathways (e.g., cAMP-mediated and protein kinase A signaling pathways) altered by adolescent drinking, with 3-fold more genes up-regulated than down-regulated in the alcohol group. The cAMP-mediated signaling system was also up-regulated in the CeA of the alcohol group. Weighted gene co-expression network analysis indicated significant G-protein coupled receptor signaling and transmembrane receptor protein kinase signaling categories in the Acb-sh and CeA, respectively. Overall, the results of this study indicated that binge-like alcohol drinking by adolescent P rats is differentially altering the expression of genes in the Acb-sh and CeA, some of which are involved in intracellular signaling pathways and may produce changes in neuronal function.

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Keywords

alcohol-preferring rat; adolescent binge drinking; nucleus accumbens-shell; central nucleus of the amygdala; gene expression

1. Introduction

Alcohol use often begins in the second decade of life, with the first use of alcohol typically occurring in early adolescence (13–14 years of age; Faden 2006). Results from the National Longitudinal Alcohol Epidemiological Survey indicated that individuals initiating alcohol use before 14 years old had a 4-fold higher rate of lifetime alcohol dependence than individuals that initiated use after the age of 20 (Grant and Dawson 1997). Moreover, over half the adolescents interviewed in other studies reported binge drinking episodes (Andersson et al., 2007; Mohshouwer et al., 2008). Twin data suggest a linear relationship between age of first use and rate of alcohol dependence, and suggest common genetic influences on age of initiation of alcohol use and alcohol dependence (Sartor et al, 2009).

Studies carried out with the alcohol-preferring (P) rat indicated that alcohol drinking during adolescence resulted, when tested during adulthood, in a quicker acquisition of operant self-administration of ethanol, greater resistance to extinction, and greater relapse drinking (Rodd-Henricks et al., 2002), suggesting a higher probability of developing alcohol addiction. These results also suggest that persisting neuronal adaptations occurred following adolescent alcohol drinking.

Examining changes in gene expression resulting from chronic ethanol drinking could provide clues toward identifying genes and gene networks involved in maintaining high alcohol drinking behavior. There have been several studies that applied genomics analyses to examining the effects of ethanol in rodent models (Bell et al., 2009; Kerns et al., 2005; McBride et al., 2010; Mulligan et al., 2006, 2011; Rodd et al., 2008; Saito et al., 2002, 2004; Tabakoff et al., 2009; Treadwell and Singh, 2004). The results of these studies indicated that differences in gene expression between alcohol-drinking rodents and water controls could be detected in several brain regions, and these differences could alter neuronal function.

Animal models have been used to study the influence of genetic factors on the effects of alcohol and on alcohol drinking behavior (reviewed in Bell et al., 2005; McBride and Li, 1998; Murphy et al., 2002). Studies on rats and mice have the advantages of allowing control of ethanol exposure and access to fresh tissues at any time. Kerns et al. (2005) reported that acute i.p. ethanol injections altered expression of genes involved in glucocorticoid signaling, neurogenesis, myelination, neuropeptide signaling, and retinoic acid signaling in three brain regions of C57BL/6J and DBA/2J mice. Rodd et al. (2008) reported that operant ethanol self-administration altered gene expression in the Acb and amygdala of inbred P rats. Another study (Bell et al., 2009) reported gene expression changes in the Acb of P rats following chronic 24-h free-choice ethanol drinking; significant differences in expression of genes involved in intracellular signaling pathways and transcription factors were found between the alcohol and water groups.

Evidence supports the involvement of the Acb in mediating ethanol drinking (reviewed in Koob et al., 1998; McBride and Li, 1998). In particular, the shell portion of the Acb is involved in supporting reinforcement (Ikemoto et al., 1997) and reinforcing effects of drugs of abuse (reviewed in Kalivas et al., 1993). The central nucleus of the amygdala (CeA) has also been implicated in mediating the effects of ethanol (Koob and Le Moal, 2008; McBride, 2002) and dependence-induced alcohol drinking (Roberts et al., 1996). Few studies on

changes in gene expression within discrete regions of the extended amygdala following alcohol drinking have been undertaken. McBride et al. (2010) reported the effects of binge-like alcohol drinking by adult male P rats on changes in gene expression within two regions of the extended amygdala, i.e., the Acb-shell (sh) and central nucleus of the amygdala (CeA). Overall, the results of this study indicated that binge-like alcohol drinking produced region-selective changes in expression of genes that could alter transcription, synaptic function and neuronal plasticity.

There have been few studies examining the effects of adolescent binge drinking on changes in gene expression within the CNS. This is mainly due to the amount of time involved in training most rodents to consume pharmacologically relevant amounts of ethanol. Since the adolescent window is approximately 10–14 days, it is difficult to establish stable ethanol intakes that produce relevant blood levels of ethanol. One study, examining the effects of i.g. administration of ethanol on changes in gene expression (Coleman et al., 2011), reported reduced levels of adult neurotransmitter gene expression, particularly of cholinergic genes.

Because of the involvement of the extended amygdala in regulating alcohol drinking, it would be important to determine the effects of adolescent alcohol drinking on gene expression changes within this system. A peri-adolescent binge-like alcohol drinking procedure has been developed, using P rats, that produces blood ethanol levels of approximately 100 mg% after 60 min (Bell et al., 2011). Therefore, the present study was undertaken to determine the effects of binge-like alcohol drinking by P rats during adolescence on changes in gene expression in the Acb-shell and CeA. The hypothesis to be tested is adolescent alcohol binge drinking will produce changes in the expression of genes associated with neuronal function and synaptic plasticity.

2. Method

2.1. Animals

Subjects were peri-adolescent (28 days old at the start of the experiment) male selectively-bred P rats from the 69th generation (n = 20 rats). The rats were single-housed on a reverse 12 h/12 h dark-light cycle (light off at 0900 hr). Animals had *ad libitum* access to food and water. The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996).

2.2. Binge-like drinking procedure

Starting at 28 days of age (PND 28) P rats (n = 10) were given concurrent access to 15 and 30% ethanol for three 1-h sessions each day during the night cycle, as previously described (Bell et al., 2011). Sessions were conducted 5 days each week (no ethanol on weekends). Water and food were always available. Rats were killed by decapitation 3 h after the 1st ethanol access session on the 15th day of drinking (when rats were 49 days old). This 3-h time-point was selected in an attempt to maximize the response to alcohol on the expression of genes in tissue from rats that have a history of repeated adolescent binge drinking. A water control group (n = 10) was killed by decapitation at the same time. Brains were quickly removed and frozen in isopentane in dry ice. Brains were stored at −80° C until they were prepared for sectioning and micro-punching.

2.3. Sample collection and microarray procedure

On the day of preparation of micro-punch samples, brains (n = 10 per group) were transferred to a cryostat set at −6 to −10° C at least 2 h prior to sectioning. Sections (300 μm) were obtained and transferred to glass slides that had been pre-cooled in the cryostat. Micro-punch sampling was done on a frozen stage (−25 to −35° C) with an anatomic microscope equipped with a cool microscope lamp. The stereotaxic atlas of Paxinos and Watson (1998) was used to identify the Acb-sh and CeA. Micro-dissection needles (Fisher Scientific) with an inner diameter of 0.77 mm were used to obtain both regions. This inner diameter fits within the entire region and minimizes contamination from adjacent tissue. Punches are taken bi-laterally from 2–3 sections. A different fresh sterile micro-punch needle was used for each animal. After withdrawing the micro-punch sample, a distinct demarcated hole remained; this hole was used to validate the micro-dissection method. All equipment used to obtain tissue was treated with RNAse Zap (Ambion, Inc. Austin, TX) to prevent RNA degradation. A second trained individual independently verified the quality of the micro-punch dissections.

The micro-punched samples were immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's protocol, but with twice the suggested ratio of Trizol to tissue (Edenberg et al., 2005). Ethanol precipitated RNA was further purified through RNeasy® columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. The yield, concentration and purity of the RNA were determined by running a spectrum from 210 to 350 nm, and analyzing the ratio of large and small ribosomal RNA bands using an Agilent Bioanalyzer. Yields, purity and quality of the RNA were excellent; RNA integrity numbers (RIN) averaged 8.5 for the samples, showing little or no degradation.

Separate preparations of total RNA were made for the Acb-sh and CeA from each animal. Samples were not pooled. Standard Affymetrix protocols (GeneChip® 3'IVT Express Kit starting with 50 ng of total RNA) were used to synthesize biotinylated cRNA, using the Affymetrix kits for cDNA synthesis, *in vitro* transcription and sample cleanup. The fragmented, biotinylated cRNA from each independent sample was mixed into 300 μl of hybridization cocktail, of which 200 μl were used for each sample. Hybridization was carried out for 17 h at 42°C. Samples were hybridized to the Affymetrix Rat Genome 230 2.0 GeneChips. Washing and scanning of the GeneChips were carried out according to standard protocols, as previously described (Edenberg et al., 2005; McClintick et al., 2003).

To minimize potential systematic errors, all stages of the experiment were balanced across experimental groups. That is, equal numbers of animals in each group were sacrificed within the same 2-h time frame each day, and equal numbers of RNA preparations from the 2 groups were processed through the labeling, hybridization, washing and scanning protocols on a given day, in a counterbalanced order, using premixes of reagents.

2.4. Statistical and bioinformatics analysis of microarray data

Each GeneChip® was scanned using an Affymetrix Model 3000 scanner and underwent image analysis using Affymetrix GCOS software. Microarray data are available from the National Center for Biotechnology Information's Gene Expression Omnibus under accession GSE49042.

Raw Cel files were imported into the statistical programming environment R (R: A language and environment for statistical computing Ver 2.2.0; R Foundation for Statistical Computing, 2005) for further analysis with tools available from the Bioconductor Project (Gentleman et al., 2004). Expression data for the 20 arrays from each of the two regions

were normalized and converted to log(2) using the Robust Multichip Average (RMA) method (Irizarry et al., 2003) implemented in the Bioconductor package RMA. As a standardization step to facilitate later comparisons with other experiments, expression levels were scaled such that the mean expression of all arrays was $\log_2(1000)$. As we were primarily concerned with identifying genes that could be subjected to further bioinformatic analysis, all probe sets currently annotated by Affymetrix as “expressed sequence tags” or whose gene names contain the words “riken”, “predicted”, or “similar to” were filtered out. We next filtered out probe sets that were not detectable above background in our samples; this has been shown to reduce noise in microarray experiments (McClintick and Edenberg, 2006). Probe sets that did not have at least 25% of samples with normalized scaled expression greater than 64 were not analyzed. Linear modeling to calculate gene-wise p-values for the contrasts of the ethanol group versus water group was performed using the package Limma (Smyth, 2004). Probe sets were considered to be statistically significant at FDR = 0.2 (calculated according to Storey et al., 2004).

Testing for over-representation of Gene Ontology (GO) biological processes (Harris et al., 2004; Ashburner et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories was performed using the Bioconductor package GOstats (Gentleman, 2004). Briefly, for each gene set tested, a list of unique Entrez-Gene identifiers was constructed. This list was then compared to the list of all known Entrez-Gene identifiers that are represented on the Affymetrix chipset Rat Genome 230 2.0. Identification of over-represented GO categories was then accomplished within GOstats using the hypergeometric distribution. GO and KEGG categories were called significant at $p < 0.05$.

Genes differentially expressed at FDR = 0.2 were uploaded into Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). Genes were identified by their Affymetrix probe set id and then mapped to their corresponding objects in the Ingenuity® Knowledge Base. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity.

To provide a more global network analysis, a weighted gene co-expression network analysis (WGCNA) was also conducted (Zhang and Horvath, 2005), using the Bioconductor (Gentleman et al., 2004) package WGCNA (Langfelder and Horvath, 2008, 2012) within R (R: A language and environment for statistical computing Ver 2.15.0; R Foundation for Statistical Computing, 2013). Briefly, gene expression data of named genes were rank-ordered according to their ascending p-values obtained from traditional t-testing of the two experimental groups. For WGCNA, default values, including the use of the power function with power β, were used for all functions with the exception that signed correlation coefficients were used. A cutoff of $p \leq 0.10$ was used to select genes for enrichment in networks that met the criteria of legitimacy for scale free topology (Zhang and Horvath, 2005). Resultant modules were tested for enrichment of various categories of genes using Fisher's Exact Test ($p \leq 0.05$) plus enrichment by cell type (Cahoy et al., 2008). Modules that satisfied these two enrichment criteria were tested for significant ($p \leq 0.01$) GO biologic process categories (Harris et al., 2004; Ashburner et al., 2000). Only significant GO categories with 40 or more genes are presented.

3. Results

3.1. Ethanol intakes

Scheduled access to ethanol was initiated on PND 28 and was conducted over a 3 week period. Ethanol intakes averaged approximately 2.5–3 g/kg for each of the 3 daily sessions

over the 15-day drinking period (Fig. 1). The highest ethanol intakes were observed during the 1st week; daily ethanol intakes were approximately 8 g/kg/day during the 2nd and 3rd week of drinking (Fig. 1).

3.2. Changes in gene expression in the Acb-sh and CeA

In the Acb-sh, there were a total of 9,059 named genes and, in the CeA, there were a total of 9,322 named genes. In the Acb-sh, there were 306 probe sets that significantly differed between the ethanol and water groups, 163 of which were named genes, representing 154 unique genes (Table 1). For the CeA, there were 335 probe sets that significantly differed between the ethanol and water groups, 190 of which were named genes, representing 182 unique gens (Table 1).

In the Acb-sh, there were 154 unique named genes that were differentially altered by adolescent alcohol drinking (Supplemental Table A). Approximately 65% of these genes had a fold-change of 1.15 or higher, and 80 genes (52%) were down-regulated. In the CeA, there were 182 unique named genes that were differentially altered by adolescent alcohol drinking (Supplemental Table B). Approximately 70% of these genes had a fold-change of 1.15 or higher, and 84 genes (46%) were down-regulated. There were 26 genes in common between the 2 regions that were significantly altered by adolescent alcohol drinking (Table 2). All 26 genes had the same directional change in both regions, with nearly identical magnitudes of change.

3.3. Bioinformatics analyses of changes in gene expression

GO analysis indicated there were 7 and 6 significant biological processes categories with 10 or more genes that differed between the water and ethanol groups in the Acb-sh and CeA, respectively (Table 3). Several of the categories in the Acb-sh involved developmental processes and cell proliferation, but none of these appeared to be directly related to neuronal development. One category (regulation of anatomical structure morphogenesis) may relate to neuronal morphological alterations. On the other hand, in the CeA, there were several categories that could be related to neuronal function, i.e., cell morphogenesis involved in neuron differentiation, neuron projection morphogenesis, positive regulation of cellular component organization, and signal release.

KEGG analysis indicated 5 significant categories in the Acb-sh, containing 5 or more genes; in the CeA, there was only 1 category that had this number of genes (Table 3). The one category in the CeA (Neuroactive ligand-receptor interaction) was also found in the Acb-sh.

Ingenuity® pathways analysis indicated 22 significant pathways with 5 or more genes that were altered in the Acb-sh by ethanol drinking, whereas only 4 canonical pathways were observed in the CeA (Table 4). In the Acb-sh, several major intracellular signaling pathways (i.e., cAMP-mediated, glucocorticoid receptor, G-protein coupled receptor and protein kinase A signaling pathways) were altered by adolescent alcohol drinking. Although cAMP-mediated signaling was observed for both regions, there was only one gene in common within this pathway, i.e., *Dusp1*. In all 4 signaling pathways in the Acb-sh, there were approximately 3-fold more genes with higher expression in the ethanol group compared to control values. In the cAMP-mediated signaling pathway in the CeA, 5 of the 6 genes were up-regulated in the alcohol group.

For the WGCNA, only by using a very liberal p-value of 0.10 as the cutoff for gene inclusion (2193 genes in the Acb-shell; 2395 genes in the CeA) did the resultant networks meet the criteria for scale free topology (Zhang & Horvarth, 2005). Modular colors indicate clusters of highly interconnected genes and are randomly assigned by the program. In the

Acb-sh, the Blue module (neuronal enrichment) contained 21 significant GO categories with 40 or more genes; in the CeA, the Yellow module contained one category and the Turquoise module (astrocytes and oligodendrocytes enrichment) contained 19 GO categories with 40 or more genes (Table 5). There were no categories that were similar between the Acb-sh and CeA. However, there were two signaling pathways that could alter neuronal function in each region, the G-protein coupled receptor signaling pathway in the Acb-sh and the transmembrane receptor protein tyrosine kinase signal pathway in the CeA.

3.4. Validation of key genes

Sufficient RNA was available in the Acb-sh samples to validate a few key genes using qRT-PCR. Five genes were selected (Table 6); these genes were significantly different in both the Acb-sh and CeA between the alcohol drinking and water control groups and had a fold-change of approximately 1.4 or higher in the Acb-sh. There was excellent agreement between the qRT-PCR values and the values obtained with the microarray procedure (Table 6).

4. Discussion

The major findings of this study are that(a) adolescent male P rats will consume 8–10 g ethanol/kg body wt/day with 3 daily binge-like drinking episodes; (b) significant changes in gene expression were found in both the CeA and Acb-sh, with 26 genes in common between the two regions; (c) there was little overlap between the 2 regions in the GO and KEGG pathways altered by adolescent binge-like drinking; and (d) adolescent alcohol drinking produced changes in several intracellular signaling pathways in both regions.

The average ethanol intakes in the present study (approximately 8 g/kg/day) were comparable to the daily intakes previous reported for adolescent P rats using the same binge-like drinking protocols (Bell et al., 2011). Blood ethanol levels (BELs) reported in this study (Bell et al., 2011) exceeded 80 mg% and approached 100 mg% on a daily basis, indicating that the adolescent P rats were attaining intoxicating blood ethanol levels on a daily basis.

Adolescent binge-like drinking altered the expression of 154 and 182 unique named genes in the Acb-sh and CeA, respectively (Supplemental Tables A & B), with 26 genes in common (Table 2). Although the Acb-sh and CeA are both involved in regulating alcohol drinking (reviewed in Koob et al., 1998; McBride and Li, 1998; McBride 2002), these 2 regions have different inputs and intrinsic neuronal circuits from each other (Cassell et al., 1999; Meredith 1999). However, there are some similarities, e.g., both receive DA inputs from the VTA and the principal type of neuron in both regions is the medium-sized GABAergic spiny neuron (Cassell et al., 1999; Meredith 1999). The common genes altered in both regions could be the result of the actions of ethanol on the same types of neurons and/or glia, whereas the unique effects of ethanol on gene expression within each region may be a result of ethanol acting on the different inputs to each region and the unique neuronal circuitries found within each region.

The bioinformatics analyses (Tables 3–5) suggested that different biological systems were being altered by adolescent binge-like drinking within each region. GO analyses (Table 3) indicated that expression of genes involved in neuron projection morphogenesis and positive regulation of cellular component organization was significantly altered by ethanol drinking in the CeA, suggesting that adolescent binge drinking may be altering neuronal function within this region. KEGG analyses (Table 3) indicated that adolescent drinking produced changes in expression of genes involved in neuroactive ligand-receptor interaction, suggesting that ethanol may be altering synaptic transmission within both regions. In addition, KEGG analyses indicated significant changes in expression of genes involved in

ErbB and Wnt signaling pathways (Table 3). Wnt pathways are involved in multiple intracellular signaling cascades that promote neuronal survival (Kikuchi et al., 2011; Scott & Brann 2013). The ErbBs are a family of receptor tyrosine kinases that allow cells to interact with the extracellular environment and transducer signals to the nucleus for proper cellular morphogenesis and function (Wadugu and Kuhn 2012; Sanchez-Soria and Camenisch 2010). These results suggest that adolescent binge-like drinking by P rats is significantly altering intracellular signaling pathways that could alter transcription in the Acb-sh. WGCNA supported the findings of the GO and KEGG analysis and indicated alterations in intracellular signaling pathways and synaptic transmission between the ethanol and water groups (Table 5).

Ingenuity Pathway analysis revealed that many more categories were significantly altered by adolescent drinking in the Acb-sh than in the CeA (Table 4). The cAMP signaling pathways in both regions had a significant number of genes altered by adolescent drinking. In both regions, there were more genes up-regulated than down-regulated in the cAMP signaling pathways (7:2 ratio and 5:1 ratio in Acb-sh and CeA, respectively), suggesting ethanol exposure enhanced the activity of this pathway. However, there was only one gene (*Dusp1*) in common in the cAMP signaling pathway between the 2 regions, suggesting that the cellular mechanisms underlying the effects of ethanol are likely different in each region. It is noteworthy that *Dusp1* also responds to glucocorticoids and is located in ethanol preference rat QTL Alc5. Paralleling the present results, another study from our laboratory revealed chronic ethanol drinking by adult P rats resulted in increased *Dusp1* gene expression in the Acb-sh (McBride et al., 2010) but reduced gene expression in the ventral tegmental area (McBride et al., 2013).

Expression of genes involved in HMGB1 (high-mobility group box 1) signaling was also altered in both regions (Table 4). HMGB1 is a PKR-dependent, DNA-binding protein and cytokine that binds to toll-like receptors (TLRs) having pro-inflammatory properties (Kang and Tang, 2012; Yang et al., 2010). Increased activity in this signaling pathway (i.e., PKR-HMGB1-TLR) may be a response to chronic high brain levels of ethanol produced by adolescent binge-like drinking with an expected influence on neuronal function, as similarly reported by Vetreno and Crews (2012; also see Crews et al., 2013).

In the Acb-sh, several signaling pathways appear to be activated by chronic adolescent binge-drinking of P rats, as indicated by the number of genes up-regulated compared to the number down-regulated in the cAMP-mediated signaling (7:2 ratio), glucocorticoid receptor signaling (7:2 ratio), RAR activation (5:1 ratio), G-protein coupled receptor signaling (8:3 ratio), and protein kinase A signaling (7:2 ratio) pathways (Table 4). The more global WGCNA was consistent with the IPA and provided support for the observed increase in G-protein coupled receptor signaling and alterations in synaptic transmission. Although the WGCNA indicated many different biological categories were altered by adolescent alcohol drinking in the CeA, there was only one signaling pathway listed, i.e., transmembrane receptor protein tyrosine kinase (Table 5). Overall, these results suggest that in the Acb-sh, and to a lesser extent in the CeA, adolescent alcohol drinking by P rats is producing significant developmental alterations in intracellular signaling pathways.

Among the 26 genes in common between the Acb-sh and CeA (Table 2), 14 were up-regulated and 12 were down-regulated in the alcohol drinking group compared to the water control group. In general, several of the genes that had higher expression levels could result in enhanced neuronal function, involving intracellular transport (*Kif15*; Hirokawa et al., 2009) and cytoskeletal organization (*Klhl23*; Wu and Gong 2004), increased Kainate (*Neto2*; Copits and Swanson 2012) and NPY (*Npy5r*) receptor function, vesicle docking (*Nsf*; Ramakrishman et al., 2012), and mitochondrial function (*Chchd5, Phb2, Slc25*; Banci

et al., 2009; Artal-Sanz and Tavernarakis 2009; Fiermonte et al., 2009). *Kif15* is synonymous with kinesin-12 a cytoskeletal motor protein, which also affects axonal growth, navigation and branching (Liu et al., 2010). *Nsf* has been reported to be up-regulated in the CeA with chronic ethanol-drinking by adult P rats (McBride et al., 2010). In this previous study, *Nsf* was identified in an Ingenuity® pathway that included multiple glutamatergic and GABAergic receptor-associated genes (McBride et al., 2010). On the other hand, the higher expression of 2 genes (*Cyr61* and *Tnfrsf11b*; Chen and Lau 2009; Hope et al., 2010) indicate that the level of alcohol drinking may be producing neuro-inflammation. *Cyr61* is synonymous with CCN1 an extracellular matrix protein involved in Fas-mediated apoptosis (Juric et al., 2009). In addition, the *Npy5r* gene has been reported to be associated with both alcohol and cocaine dependence in human studies (Wetherill et al., 2008).

Many of the genes that had lower expression levels in the alcohol than water group could result in altered transcription (*Chd7, Klf3, Mga, Mtf1, Sp1*; Kim and Layman 2011; Moore et al., 2011; Hurlin et al., 1999; Giedroc et al., 2001; Lomberk and Urrutia 2005), and reduced response to oxidative (*Angptl4*; Zhu et al., 2012) or other cellular (*C1s, Cdkn1a, Limd1*; Schumaker et al., 1986; Mergenthaler et al., 2013; Foxler et al., 2012) stresses. These results suggest that adolescent binge-like alcohol drinking may have reduced the capabilities of many of the neurons and glia in the Acb-sh and CeA to adequately regulate the levels of reactive oxygen species. Interestingly, *Limd1* was identified as a high alcohol consumption candidate gene in a meta-analysis study with mice (Mulligan et al., 2006).

Adolescent binge-like exposure reduced basal α-MSH immuno-reactivity in the CeA (Lerma-Cabrera et al., 2013). Another study indicated that adolescent consumption of a sweetened alcohol solution by female Wistar rats reduced the number of CRF immuno-reactive cells in the CeA (Karanikas et al., 2013). A third study (Alaux-Cantin et al., 2013), using binge-like ethanol administration, reported lower c-fos immunoreactivity in the Acb and enduring alterations in the expression of *Penk* and *Slc6a4*. These results are consistent with the results of the present study indicating that the Acb and CeA may be vulnerable to the effects of ethanol during adolescence. In addition, the WGCNA indicated a significant number of GO categories associated with astrocytes and oligodendrocyte functions. Alterations in glial function have been produced by alcohol exposure and may contribute to alcohol drinking behavior (Lee et al., 2013; Nam et al., 2012; Paul & Medina 2012).

A previous study (McBride et al., 2010) examined the effects of adult binge-like drinking on gene expression in the Acb-sh and CeA of adult P rats. A similar procedure of 3 one-h sessions per day during the dark cycle for 5 consecutive days each week was used in this previous study. The ethanol intakes and BELs for the adolescent and adult P rats were similar (Bell et al., 2011; McBride et al., 2010). In the Acb-sh, although there were 4 genes (*Hhex, Nyw1, Usp18, Zfp36l1*) in common between the present study (Supplemental Table A) and the previous adult study (McBride et al., 2010), the direction of change was not similar. In the CeA, there were 8 genes in common between the adult and adolescent findings, with 6 genes (*Asrd, Cxcl12, Nsf, Tfrc, Ube2b, Zc3h8*) changing in the same direction in both studies and only 2 genes (*Ier2, Nfil3*) changing in opposite directions. The overall general paucity of common genes between the adult and adolescent findings in the Acb-sh and CeA may illustrate the unique effects that ethanol has during the peri-adolescent period.

Within the Acb-sh, there were several biological systems (using GO, KEGG and IPA analyses) that were significantly changed following both peri-adolescent and adult binge like drinking. These included glucocorticoid receptor (GR) signaling, Wnt signaling, regulation of anatomical structure morphogenesis, and cancer. Changes in GR and Wnt signaling could alter the transcription of multiple genes affecting a variety of cellular functions (Ratman et

al., 2013; Kikuchi et al., 2011). Even though alcohol drinking by adolescent and adult P rats altered similar biological systems, the mechanisms underlying alcohol's actions appear to be different since there are so few genes in common that were changed in both studies.

In the CeA, there were some common biological systems that overlap between the present study and the adult study (McBride et al., 2010), these included neuroactive ligand-receptor interaction (Table 3), neuron projection morphogenesis (Table 3) and positive regulation of cell component organization (Table 3). In the adult, the genes associated with neuron projections and regulation of cell organization were mainly up-regulated suggesting an overall positive effect of binge drinking on these biological systems.

Overall, the effects of binge drinking by P rats on gene expression appear to be age and region dependent. Although there are some biological systems in common between the effects of adolescent vs. adult ethanol intake, there are few genes in common within these systems, suggesting different mechanisms of action and their associated outcomes at the 2 different ages. Moreover, there was only one biological system in common between the 2 regions, which likely reflects the different inputs to and neuronal circuits within the Acb-sh vs, the CeA.

The effects of repeated i.g. administration of ethanol (5 g/kg once per day for 10 consecutive days) during adolescence (PND 28 – 37) were studied on changes of gene expression in the whole brain of C57BL/6 mice (Coleman et al., 2011). In this study, RT-PCR arrays targeting genes for different peptide, cholinergic, monoamine, GABA and glycine receptors were used. In general, measurements taken on PND 38 indicated reduced expression of genes for these receptors in the ethanol-treated group compared to the controls. These findings are in contrast to the present results where expression of genes for different receptors were increased in the Acb-sh (*Adora2b*, *Adrb2*, *Npy5r*; Supplemental Table A) and CeA (*Gabrb3*, *Grm7*, *Hrh3*, *Htr1d*, *Npy5r*, *Tfrc*; Supplemental Table B) of the binge drinking group vs. the water control. In no case was the expression of genes for any receptor significantly decreased by adolescent binge-like drinking in the present study. The contrast in the results between the present study and the findings of Coleman and colleagues (2011) could be due to a combination of factors: (a) i.g. bolus administration of ethanol, which produced BELs of ~280 mg% vs. binge-like drinking, which produced BELs of ~100 mg%; (b) whole brain vs. discrete regions; and/or (c) inbred mouse vs. selectively bred rat.

In summary, adolescent binge-like alcohol drinking (ethanol intakes of 2.5 – 3.0 g/kg/session) by P rats produced effects on the expression of genes involved in multiple biological cellular pathways in the Acb-sh and CeA. These pathways included several important intracellular signaling systems, e.g., cAMP-mediated, glucocorticoid receptor signaling, protein kinase A signaling, etc, with more genes up-regulated than down-regulated in the alcohol group. Some of the changes in gene expression produced by adolescent binge-like drinking may be unique to the selectively bred P rat, and/or unique to this stage of development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We examined binge-like alcohol drinking by adolescent alcohol preferring (P) rats
- Gene expression within two regions of the extended amygdala were determined
- cAMP signaling genes were up-regulated by adolescent alcohol drinking in both regions
- Genes for several intracellular signaling pathways were altered by alcohol drinking

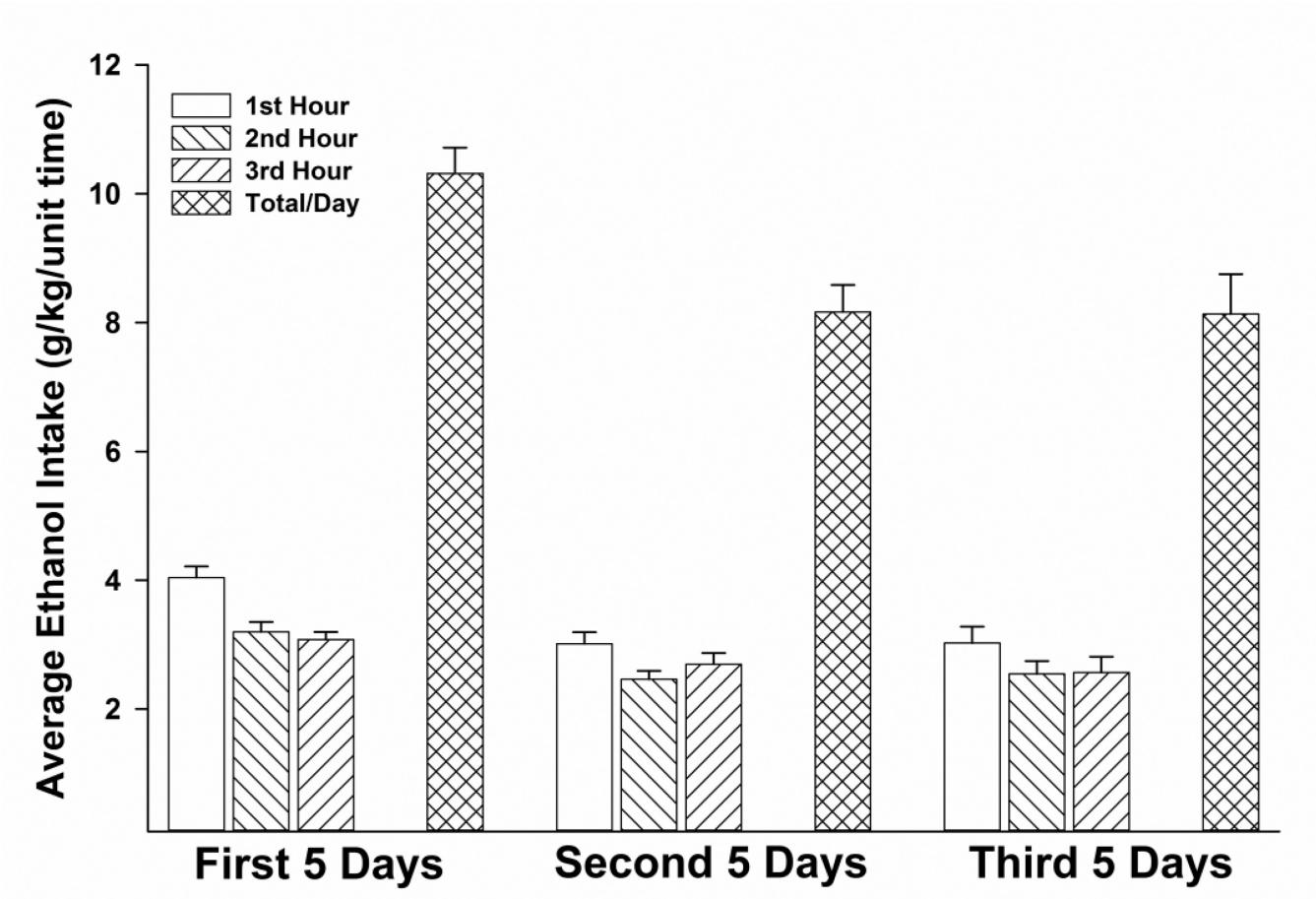

Fig. 1.

Ethanol intakes (g/kg) by peri-adolescent P rats averaged over each 5-day period for each of the three one-h periods with concurrent access to 15 and 30% ethanol (with water freely available) during the dark cycle. Ethanol was given 5 consecutive days each week over the 15 days of access. The average total per day for the 3 one-h sessions is also shown. Data are the means ± SEM; n = 10).

Table 1

Summary of significant probe sets in the Acb-sh and CeA of adolescent alcohol drinking and control P rats

Item	Acb-sh	CeA
FDR threshold - Storey	0.2	0.2
Probe sets after A/P filter	22027	22679
total significant probe sets	306	335
total significant ESTs	143	145
total significant probe sets of named genes	163	190
total unique named genes	154	182

Table 2

List of genes that were significantly altered by adolescent alcohol drinking in both the Acb-sh and CeA

Symbol	Gene Description	F-C Acb-sh	F-C CeA
Angptl4	angiopoietin-like 4	−1.19	−1.21
Atic	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	−1.23	−1.25
C1s	complement component 1, s subcomponent	−1.33	−1.42
Ccdc49	coiled-coil domain containing 49	1.37	1.29
Cdkn1a	cyclin-dependent kinase inhibitor 1A	−1.26	−1.55
Chchd5	coiled-coil-helix-coiled-coil-helix domain containing 5	1.25	1.26
Chd7	chromodomain helicase DNA binding protein 7	−1.16	−1.30
Cyr61	cysteine-rich, angiogenic inducer, 61	3.83	1.56
Dusp1	dual specificity phosphatase 1	1.88	1.57
Fam168a	family with sequence similarity 168, member A	−1.25	−1.28
Ier2	immediate early response 2	1.7	1.26
Kif15	kinesin family member 15	1.39	1.45
Klf3	Kruppel-like factor 3 (basic)	−1.13	−1.19
Klhl23	kelch-like 23 (Drosophila)	1.15	1.11
Limd1	LIM domains containing 1	−1.14	−1.20
Mga	MAX gene associated	−1.3	−1.34
Msn	Moesin	−1.16	−1.26
Mtf1	metal-regulatory transcription factor 1	−1.13	−1.12
Neto2	neuropilin (NRP) and tolloid (TLL)-like 2	1.2	1.23
Npy5r	neuropeptide Y receptor Y5	1.23	1.36
Nsf	N-ethylmaleimide-sensitive factor	1.15	1.26
Phb2	Prohibitin 2	1.24	1.17
Slc25a23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23	1.14	1.20
Sp1	Sp1 transcription factor	−1.13	−1.14
Tfrc	transferrin receptor	1.14	1.15
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	1.63	1.35

F-C = fold change; negative sign indicates decreased expression in the alcohol drinking group vs. the water control group.

Table 3

Significant GO biological processes categories with 10 or more genes or significant KEGG categories with 5 or more genes in the Acb-sh and CeA of adolescent alcohol drinking vs. control P rats

GO Cat. ID	Term	P-value	Count	Size
Acb-sh				
GO:0001568	blood vessel development	0.000	19	260
GO:0048534	hemopoietic or lymphoid organ development	0.003	11	265
GO:0001701	in utero embryonic development	0.000	14	200
GO:0008285	negative regulation of cell proliferation	0.007	10	262
GO:0008284	positive regulation of cell proliferation	0.001	12	297
GO:0022603	regulation of anatomical structure morphogenesis	0.001	11	232
GO:0014070	response to organic cyclic compound	0.003	10	231
CeA				
GO:0007017	microtubule-based process	0.012	10	230
GO:0023061	signal release	0.001	12	214
GO:0035295	tube development	0.015	11	276
GO:0048667	cell morphogenesis involved in neuron differentiation	0.018	10	247
GO:0048812	neuron projection morphogenesis	0.010	11	258
GO:0051130	positive regulation of cellular component organization	0.048	10	290
KEGG Cat. ID	**Term**	**P-value**	**Count**	**Size**
Acb-sh				
4012	ErbB signaling pathway	0.004	5	66
4080	Neuroactive ligand-receptor interaction	0.01	6	123
4310	Wnt signaling pathway	0.02	5	100
5200	Pathways in cancer	0.02	8	207
5210	Colorectal cancer	0.0001	6	50
CeA				
4080	Neuroactive ligand-receptor interaction	0.03	6	137

Table 4

List of significant Ingenuity canonical pathways with 5 or more genes in the AcB-sh and CeA between the adolescent alcohol drinking and control group

Ingenuity Canonical Pathways	p-value	Ratio	Molecules
Acb-sh			
Ceramide Signaling	0.00005	0.07	S1PR3, FOS, JUN, S1PR2, S1PR1, TNFRSF11B
cAMP-mediated signaling	0.00007	0.04	S1PR3, AKAP2/PALM2-AKAP2, CAMK2A, MAPK1, DUSP1 DUSP6, S1PR1, ADORA2B ADRB2
IGF-1 Signaling	0.00014	0.06	FOS, JUN, CTGF, MAPK1 CYR61, GRB10
Glucocorticoid Receptor Signaling	0.00030	0.03	BCL2L1, FOS, NFAT5, JUN MAPK1, DUSP1, CDKN1A SMAD4, ADRB2
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.00047	0.06	FOS, NFAT5, JUN, MAPK1 SMAD4
Human Embryonic Stem Cell Pluripotency	0.00065	0.04	S1PR3, NTRK2, S1PR2, S1PR1 SMAD4, LEF1
PI3K Signaling in B Lymphocytes	0.00065	0.04	FOS, NFAT5, JUN, ATF3 CAMK2A, MAPK1
HMGB1 Signaling	0.00100	0.05	FOS, JUN, SP1, MAPK1 TNFRSF11B
Molecular Mechanisms of Cancer	0.00186	0.02	BCL2L1, FOS, JUN, CAMK2A MAPK1, CDKN1A, SMAD4 LEF1, NOTCH1
Pancreatic Adenocarcinoma Signaling	0.00186	0.04	BCL2L1, MAPK1, CDKN1A SMAD4, NOTCH1
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.00204	0.03	FOS, NFAT5, JUN, MAPK1 SMAD4, LEF1, TNFRSF11B
PTEN Signaling	0.00295	0.04	BCL2L1, NTRK2, MAPK1 CDKN1A, PREX2
RAR Activation	0.00302	0.03	FOS, JUN, MAPK1, DUSP1 PNRC1, SMAD4
G-Protein Coupled Receptor Signaling	0.00316	0.02	S1PR3, S1PR2, CAMK2A MAPK1, DUSP1, ELTD1 DUSP6, S1PR1, ADORA2B NPY5R, ADRB2
Colorectal Cancer Metastasis Signaling	0.00347	0.03	BCL2L1, FOS, JUN, MAPK1 DCC, SMAD4, LEF1
Protein Kinase A Signaling	0.00468	0.02	AKAP2/PALM2-AKAP2 NFAT5, CAMK2A, MAPK1 DUSP1, DUSP6, DCC, SMAD4 LEF1
Aryl Hydrocarbon Receptor Signaling	0.00575	0.03	FOS, JUN, SP1, MAPK1 CDKN1A
B Cell Receptor Signaling	0.00851	0.03	BCL2L1, NFAT5, JUN CAMK2A, MAPK1
Systemic Lupus Erythematosus Signaling	0.00955	0.02	FOS, NFAT5, JUN, MAPK1 PRPF4B, PRPF38B
Acute Phase Response Signaling	0.01202	0.03	FOS, JUN, MAPK1, C1S TNFRSF11B
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.01230	0.02	FOS, NFAT5, JUN, CAMK2A MAPK1, LEF1, TNFRSF11B
Huntington's Disease Signaling	0.03311	0.02	NSF, BCL2L1, JUN, SP1 MAPK1
CeA			
ATM Signaling	0.0003	0.08	RAD51, CDKN1A, CCNB2, BID CCNB1
HMGB1 Signaling	0.0023	0.05	SP1, IFNGR2, RHOJ TNFRSF11B, PLAT
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.0026	0.04	TIMP1, MYH14, IFNGR2 IGFBP5, PDGFRB, TNFRSF11B
cAMP-mediated signaling	0.0200	0.03	GRM7, DUSP1, CREM (includes EG:12916), HTR1D, HRH3 AKAP1

Table 5

List of significant GO categories with 40 or more genes in the WGCNA of the Acb-sh and CeA of adolescent alcohol drinking and control P rats

Category_ID	Term	P-value	OddsRatio	ExpCount	Count	Size
	Acb-sh (Blue)					
GO:0008015	blood circulation	0.001	1.65	42.3	61	211
GO:0006874	cellular calcium ion homeostasis	0.002	1.68	32.7	48	163
GO:0030003	cellular cation homeostasis	0.001	1.61	44.4	63	221
GO:0055082	cellular chemical homeostasis	0.000	1.51	87.1	117	434
GO:0072507	divalent inorganic cation homeostasis	0.003	1.63	36.3	52	181
GO:0070838	divalent metal ion transport	0.000	1.79	35.3	54	176
GO:0007186	G-protein coupled receptor signaling pathway	0.007	1.47	49	65	245
GO:0051325	interphase	0.004	1.71	26.9	40	134
GO:0034220	ion transmembrane transport	0.005	1.49	50.8	68	253
GO:0055065	metal ion homeostasis	0.004	1.58	39.9	56	199
GO:0035637	multicellular organismal signaling	0.001	1.45	87.9	115	438
GO:0003012	muscle system process	0.000	2.12	27.5	47	137
GO:0060537	muscle tissue development	0.002	1.6	43.9	62	219
GO:0044092	negative regulation of molecular function	0.009	1.35	78.9	98	393
GO:0072522	purine-containing compound biosynthetic process	0.005	1.68	28.7	42	143
GO:0060341	regulation of cellular localization	0.003	1.39	87.7	111	437
GO:0044057	regulation of system process	0.000	1.83	54.4	84	273
GO:1901698	response to nitrogen compound	0.010	1.31	98.1	119	489
GO:0009260	ribonucleotide biosynthetic process	0.007	1.63	28.5	41	142
GO:0023061	signal release	0.009	1.48	43.3	58	216
GO:0007268	synaptic transmission	0.010	1.38	62.9	80	315
	CeA (yellow)					
GO:0051276	chromosome organization	0.000	1.84	29.3	49	383
	CeA (Turquoise)					
GO:0001775	cell activation	0.007	1.46	46.8	63	309
GO:0002009	morphogenesis of an epithelium	0.001	1.69	35.9	54	237
GO:0006952	defense response	0.008	1.4	56.8	74	375
GO:0007155	cell adhesion	0.005	1.4	66.3	86	438

Category_ID	Term	P-value	OddsRatio	ExpCount	Count	Size
GO:0007169	transmembrane receptor protein tyrosine kinase signal pathway	0.007	1.54	34.8	49	230
GO:0008284	positive regulation of cell proliferation	0.005	1.43	57.4	76	379
GO:0009611	response to wounding	0.000	1.56	62.5	88	413
GO:0009617	response to bacterium	0.008	1.56	30.9	44	204
GO:0009968	negative regulation of signal transduction	0.002	1.5	56.1	77	372
GO:0016477	cell migration	0.008	1.35	74.5	94	492
GO:0032787	monocarboxylic acid metabolic process	0.001	1.69	37.3	56	246
GO:0035295	tube development	0.002	1.55	44.8	63	296
GO:0043066	negative regulation of apoptotic process	0.000	1.55	62.1	87	410
GO:0043085	positive regulation of catalytic activity	0.003	1.43	69.2	91	457
GO:0044255	cellular lipid metabolic process	0.000	2.1	50.4	88	336
GO:0044283	small molecule biosynthetic process	0.002	1.73	29.2	45	195
GO:0046394	carboxylic acid biosynthetic process	0.000	1.87	27.6	45	182
GO:0060548	negative regulation of cell death	0.002	1.47	66.3	89	438
GO:1901615	organic hydroxy compound metabolic process	0.000	1.84	33	53	218

Table 6

Validation with qRT-PCR of 5 genes in Acb-sh that were significantly different in both the Acb-sh and CeA between the water and EtOH groups and had a fold-change of approximately 1.4 or higher between the groups

	qRT-PCR				Microarray
Gene	**Water**	**EtOH**	**F-C**	**p-value**	**F-C**
Ccdc49	1.45 ± 0.03	1.66 ± 0.04	1.14	0.000	1.37
Cyr61	0.79 ± 0.03	4.13 ± 0.33	5.26	0.000	3.83
Dusp1	6.22 ± 0.73	7.50 ± 0.49	1.21	0.007	1.88
Kif15	1.09 ± 0.05	1.94 ± 0.14	1.78	0.000	1.39
Tnfrsf11b	0.49 ± 0.02	0.79 ± 0.01	1.62	0.000	1.63

Data are the means ± SEM (n = 10/group). F-C = fold-change